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A defined growth medium for *Clostridium difficile*

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Minimal requirements of amino acids and vitamins were determined in chemically defined medium for five strains of *Clostridium difficile*. Cysteine, isoleucine, leucine, proline, tryptophan and valine were essential amino acids for growth of *C. difficile*. Arginine, glycine, histidine, methionine and threonine enhanced growth. Biotin, pantothenate and pyridoxine were essential vitamins. A defined medium containing the minimal requirements of amino acids and vitamins produced a rapid and heavy growth which was comparable to that in modified brain heart infusion, a complex medium. Adenine was able to substitute for glycine and threonine, suggesting that the two amino acids may be utilized as precursors of purine nucleotides. The defined medium developed here will assist physiological and biochemical studies on *C. difficile*.

Keywords: *Clostridium difficile*, defined growth medium, amino acid, viramin

INTRODUCTION

Clostridium difficile causes pseudomembranous colitis and is a major aetiological agent of antibiotic-associated diarrhoea (Bartlett *et al.*, 1978a; George *et al.*, 1978; Larson *et al.*, 1978; Borriello & Larson, 1981). It produces toxin A and toxin B, which are major virulence factors (Banno *et al.*, 1984; Lyerly *et al.*, 1986, 1988; Sullivan *et al.*, 1982). Fimbriae, capsule and tissue degradative enzymes have also been considered as virulence factors (Borriello *et al.*, 1988; Strelau *et al.*, 1989; Davis & Borriello, 1990; Seddon *et al.*, 1990, 1991).

Cooked meat glucose broth (Bartlett *et al.*, 1978b), brain heart infusion (BHI) (Lyerly *et al.*, 1985) and modified BHI (m-BHI) (Honda *et al.*, 1983; Nakamura *et al.*, 1985) are widely used to obtain good growth and high toxin production of *C. difficile*. However, these media are not suitable for analysis of nutritional effects on growth and toxin production because they are neither defined nor uniform in composition. Several defined media have been used for examination of nutritional effects on production of the toxins and other virulence factors (Haslam *et al.*, 1986; Seddon *et al.*, 1991), and for isolation of the organism (Hubert *et al.*, 1981). However, little work has been performed on the minimal nutritional requirements for bacterial growth. In the present study, we determined the minimal requirements for amino acids and vitamins for growth of *C. difficile* and developed a defined medium containing the minimal organic components to produce good growth.

METHODS

Bacterial strains. Toxigenic *C. difficile* strains VPI 10463, KZ 1626, KZ 1630, KZ 1647 and KZ 1748 were used. The latter four strains were isolated in our laboratory from healthy adults or patients with antibiotic-associated diarrhoea (Nakamura *et al.*, 1982).

Preparation of media. Composition of basal defined medium (BDM) (Table 1) was based on the defined medium described by Haslam *et al.* (1986) with four modifications: 600 mg proline l^{-1}, 300 mg KH_2PO_4 l^{-1} and 1500 mg Na_2HPO_4 l^{-1} were added, and Na_2CO_3 was replaced with 5000 mg $NaHCO_3$ l^{-1}. BDM was sterilized by membrane filtration (Millex-HA, 0·45 μm pore size; Nihon Millipore) and distributed in 10 ml amounts in 15 × 160 mm test-tubes flushed with an O_2-free gas mixture [$H_2/CO_2/N_2$ (10:10:80, by vol.)]. The tubes were then stoppered with rubber stoppers. m-BHI (Nakamura *et al.*, 1985) was used as complex medium. All media were pre-reduced for at least 48 h before use.

Culture conditions. *C. difficile* strains were cultivated on modified cycloserine-cefoxitin-fructose-agar (CCFA) plates (Nakamura *et al.*, 1981) anaerobically for 48 h. Several colonies of each strain were transferred to a medium for subculture (see Tables 2–4) and incubated at 37 °C for 10 h. The culture was then diluted 1000-fold in pre-reduced 0·85 % (w/v) NaCl and 0·1 ml of the diluted culture was inoculated in duplicate into test media. The cultures were incubated at 37 °C. Inoculation,

Table 1. Composition of basal defined medium (BDM)

Component	Concn (mg)	Component	Concn (mg)
Amino acid		**Vitamin**	
Histidine	100	Thiamin	1
Tryptophan	100	Calcium-D-pantothenate	1
Glycine	100	Nicotinamide	1
Tyrosine	100	Riboflavin	1
Arginine	200	Pyridoxine	1
Phenylalanine	200	*p*-Aminobenzoic acid	0·05
Methionine	200	Folic acid	0·0125
Threonine	200	Biotin	0·0125
Alanine	200	B_{12}	0·005
Lysine	300		
Serine	300	**Mineral**	
Valine	300	KH_2PO_4	300
Isoleucine	300	Na_2HPO_4	1500
Aspartic acid	300	NaCl	900
Leucine	400	$CaCl_2.2H_2O$	26
Cysteine	500	$MgCl_2.6H_2O$	20
Proline	600	$MnCl_2.4H_2O$	10
Glutamic acid	900	$(NH_4)_2SO_4$	40
		$FeSO_4.7H_2O$	4
Glucose	2000	$CoCl_2.6H_2O$	1
		$NaHCO_3$	5000
		Distilled water (ml)	1000

dilution and incubation were performed under the anaerobic gas mixture.

Bacterial growth. The OD_{560} of cultures was measured every 2 h during a 24 h incubation period with a Spectronic 20A spectrophotometer (Shimadzu). Mean OD_{560} values of duplicate tests are presented in Results.

RESULTS

Determination of minimal requirements for amino acids and vitamins

Growth of all five test strains reached maximum after an incubation period of 20–22 h in BDM and m-BHI. The maximum OD_{560} values were 0·80–0·88 in BDM and 0·73–0·84 in m-BHI. To identify amino acid requirements for good growth of the five strains, experiments were performed using BDM lacking a single amino acid. No growth was observed in the absence of cysteine, isoleucine, leucine, proline, tryptophan and valine (Table 2). When arginine, histidine and methionine were omitted, the growth of all strains was reduced markedly. These results indicated that the former six amino acids were essential for growth and the latter three were growth-enhancing. However, since an amino-acid-limited synthetic medium containing the six essential and three growth-enhancing amino acids failed to produce good growth (Table 3), the effects of the remaining amino acids on growth were further examined by single-amino-acid-addition experiments. For all five test strains, full growth (relative to complete BDM) was restored by addition of either glycine or threonine to the amino-acid-limited synthetic medium containing the nine other amino acids. Thus, we concluded that glycine and threonine belonged to the group of growth-enhancing amino acids.

Minimal requirements for the essential and growth-enhancing amino acids were quantified using strain VPI 10463. Amino-acid-limited synthetic media containing the six essential and five growth-enhancing amino acids,

Table 2. Growth of *C. difficile* strains in BDM lacking a single amino acid*

Amino acid omitted	Maximum OD_{560} of:†				
	VPI 10463	KZ 1626	KZ 1630	KZ 1647	KZ 1748
Cysteine	–	–	–	–	
Isoleucine	–	–	–	–	–
Leucine				–	–
Proline	–	–	–		–
Tryptophan	0·01	0·05	0·06	0·05	0·03
Valine	–	–	–	–	–
Arginine	0·40	0·50	0·42	0·48	0·40
Histidine	0·23	0·56	0·45	0·48	0·41
Methionine	0·12	0·06	0·27	0·26	0·29
Other nine amino acids‡	0·84–0·89	0·79–0·89	0·80–0·84	0·80–0·85	0·78–0·81
None	0·88	0·84	0·83	0·83	0·80

–, No visible growth.

* A subculture of each test strain in BDM was inoculated in test media.

† Maximum OD_{560} value during a 24 h incubation period.

‡ Alanine, aspartic acid, glutamic acid, glycine, lysine, phenylalanine, serine, threonine and tyrosine.

Table 3. Effects of glycine and threonine on growth of *C. difficile* strains in a synthetic medium containing nine amino acids*

Amino acid added†	Maximum OD_{560} of:				
	VPI 10463	KZ 1626	KZ 1630	KZ 1647	KZ 1748
Glycine	0·80	0·75	0·76	0·70	0·80
Threonine	0·86	0·80	0·72	0·73	0·82
Other seven amino acids‡	0·03–0·08	0·31–0·58	0·11–0·27	0·15 0·36	0·23· 0·56
None	0·03	0·48	0·24	0·32	0·53

* Arginine, cysteine, histidine, isoleucine, leucine, methionine, proline, tryptophan and valine; their concentrations were those in BDM. A subculture of each test strain in a synthetic medium containing the nine amino acids was inoculated into test media.

† Concentrations of amino acids were those in BDM.

‡ Alanine, aspartic acid, glutamic acid, lysine, phenylalanine, serine and tyrosine.

Table 4. Growth of *C. difficile* strains in synthetic media lacking a single vitamin and containing 11 amino acids*

Vitamin omitted	Maximum OD_{560} of:				
	VPI 10463	KZ 1626	KZ 1630	KZ 1647	KZ 1748
Biotin	0·03	0·03	0·03	0·05	0·03
Calcium-D-pantothenate	0·01	0·01	0·01	0·01	0·02
Pyridoxine	0·01	0·03	0·09	0·18	0·19
Other six vitamins†	0·84–0·88	0·78–0·83	0·75–0·79	0·75–0·85	0·76–0·79
None	0·85	0·80	0·77	0·76	0·78

* Eleven amino acids (mg l^{-1}) were: arginine, 100; cysteine, 500; glycine, 100; histidine, 100; isoleucine, 100; leucine, 1000; methionine, 100; proline, 800; threonine, 100; tryptophan, 100; valine, 100. A subculture of each test strain in a synthetic medium containing the 11 amino acids and vitamins in BDM was inoculated into test media.

† B_{12}, folic acid, nicotinamide, *p*-aminobenzoic acid, riboflavin and thiamin.

in which the concentration of single amino acids was varied, were prepared and bacterial growth in these media was measured. The minimal concentrations of the amino acids required for optimal growth were as follows (mg l^{-1}): arginine, 10; cysteine, 400; glycine, 20; histidine, 5; isoleucine, 10; leucine, 500; methionine, 10; proline, 400; threonine, 40; tryptophan, 5; valine, 20. Based on these findings, we determined practical amino acid concentrations for further experiments as follows (mg l^{-1}): arginine, 100; cysteine, 500; glycine, 100; histidine, 100; isoleucine, 100; leucine, 1000; methionine, 100; proline, 800; threonine, 100; tryptophan, 100; valine, 100.

To identify vitamin requirements for good growth of the five strains, experiments in which single vitamins were omitted were performed with a defined medium containing the six essential and five growth-enhancing amino acids, and vitamins used in BDM. The maximum OD_{560} values were 0·03–0·05 in the medium lacking biotin, 0·01–0·02 in the medium lacking pantothenate and 0·01–0·19 in the medium lacking pyridoxine (Table 4). Omission of the other six vitamins did not reduce bacterial growth. These results indicated that biotin, pantothenate and pyridoxine were essential vitamins for growth. Minimal requirements for the essential vitamins were quantified using strain VPI 10463. Vitamin-limited synthetic media containing the three essential vitamins, in which the concentration of a single vitamin was varied, were prepared and bacterial growth in these media was measured. The minimal concentrations of the vitamins required for optimal growth were as follows (μg l^{-1}): biotin, 2; calcium-D-pantothenate, 400; pyridoxine, 20. Based on these findings, we determined practical vitamin concentrations for further experiments as follows (μg l^{-1}): biotin, 10; calcium-D-pantothenate, 1000; pyridoxine, 100.

Bacterial growth in a new defined medium

A defined medium containing the minimal amino acids and vitamins at the practical concentrations was designated *C. difficile* minimal medium (CDMM). Growth of all

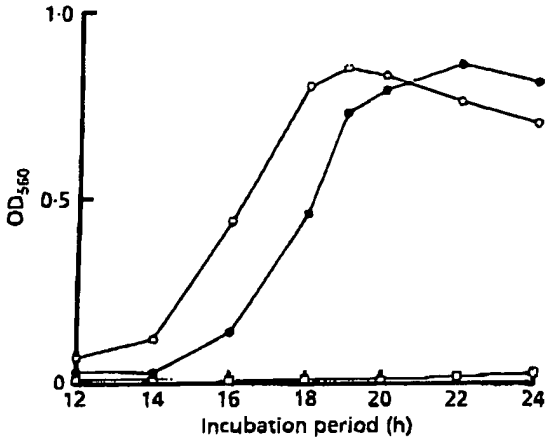

Fig. 1. Substitution of adenine for glycine and threonine for growth of *C. difficile* strain VPI 10463. ○, CDMM; □, CDMM lacking glycine and threonine; ●, CDMM lacking glycine and threonine, with added adenine (30 μg l^{-1}).

five test strains in CDMM reached a maximum after 20–22 h and the maximum OD_{560} values were 0·80–0·85. No differences in growth of the strains were observed between CDMM, BDM and m-BHI.

Effect of adenine on growth

Addition of adenine produced good growth in CDMM lacking glycine and threonine, although the incubation period needed to reach maximum growth was 3 h longer than that in CDMM (Fig. 1). Adenine increased bacterial growth in a dose-dependent fashion, with a minimal concentration for optimal growth of 20 μg l^{-1}.

DISCUSSION

We attempted to determine minimal requirements for amino acids and vitamins and to develop a defined medium for good growth of *C. difficile*. Cysteine, isoleucine, leucine, proline, tryptophan and valine were essential for bacterial growth, and arginine, glycine, histidine, methionine and threonine were found to be growth-enhancing amino acids. Glycine and threonine could substitute for each other. Biotin, pantothenate and pyridoxine were essential vitamins for growth. Considering that all five test strains showed the same results, we presume that the requirements of amino acids and vitamins identified here generally hold true for *C. difficile* strains.

Haslam *et al.* (1986) showed which amino acids were essential for growth of *C. difficile* by the single-amino-acid-omission method, on which our experiments were based. The essential amino acids identified in the present work were identical to those in the earlier study, except for methionine. Furthermore, we were able to show that arginine, glycine, histidine, methionine and threonine were growth-enhancing amino acids by employing a quantitative analysis for bacterial growth instead of a qualitative analysis. Subsequently, we determined the minimal concentrations of the amino acids and vitamins for optimal growth. Seddon & Borriello (1989) reported a defined medium for *C. difficile*, the contents of which were different from our defined medium, CDMM. It is unclear, however, whether the amino acids and vitamins in their medium were essential for growth since there was no description of how nutritional requirements were determined. In addition, bacterial growth in their medium was very much poorer than in BHI. This may be because their defined medium did not contain the essential amino acids and vitamins identified in our study, such as isoleucine, tryptophan, biotin, pantothenate and pyridoxine. Hubert *et al.* (1981) also reported a minimal medium for isolation of *C. difficile*. The requirements for amino acids – leucine, methionine, proline, tryptophan and valine – and vitamins – biotin, pantothenate and pyridoxine – determined by them were very similar to our own results. However, as their aim was to develop a minimal medium for isolation of *C. difficile*, their method of evaluation of bacterial growth, such as a 48 h incubation period and use of agar-containing media, distinctly differed from ours. Thus, it is not appropriate to compare their results with ours. Moreover, 2% bile contained in their medium might affect determination of nutritional requirements.

Boyd *et al.* (1948) reported that 13 amino acids – arginine, cystine, glutamic acid, histidine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine – and four vitamins – biotin, pantothenate, pyridoxamine and riboflavin – were essential for growth of *Clostridium perfringens* BP6K. Fuchs & Bonde (1957) observed that 11 amino acids – arginine, aspartic acid, cysteine, glutamic acid, histidine, leucine, phenylalanine, threonine, tryptophan, tyrosine and valine – and three vitamins – biotin, pantothenate and pyridoxine – were essential for *C. perfringens* strains. Whitmer & Johnson (1988) investigated nutritional requirements of *Clostridium botulinum*. Eight amino acids – arginine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine and valine – were essential for growth of a proteolytic type B strain, Okura B, while five amino acids – isoleucine, leucine, tryptophan, tyrosine and valine – were essential for growth of nonproteolytic type E strains. Although there was variation in vitamin requirements among the strains they tested, biotin was essential for growth of the type B and E strains. To summarize these previous and present studies, it is suggested that these three pathogenic species share the nutritional requirements of isoleucine, leucine, tryptophan, valine and biotin for growth. The habitat of *C. difficile* and *C. perfringens* is the alimentary tract, while *C. botulinum* is found in soil. Interestingly, vitamin requirements of the two former species are almost identical but those of the latter are different.

Glycine is a precursor of purine nucleotides in the *de novo* pathway and threonine is also available after being converted into glycine (Sonenshein, 1993). Alternatively, adenine is reused to synthesize purine nucleotides through the salvage pathway. We found that glycine or threonine

enhanced growth of *C. difficile*, and could substitute for one another, and that adenine compensated for either amino acid. Therefore, both amino acids may be important as precursors of purine nucleotides in *C. difficile*. Serine, which is generally converted into glycine (Sonenshein, 1993), was neither essential for nor stimulated growth of *C. difficile* in our study. This finding suggests that *C. difficile* may not have a pathway to convert serine into glycine.

The defined medium developed here, CDMM, is of minimal composition to produce good growth of *C. difficile*. It will be useful for studies on the physiology, metabolism and virulence factors of the organism.

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